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Effect of Inactivation of the Arg- and/or Lys-Gingipain Gene on Selected Virulence and Physiological Properties of *Porphyromonas gingivalis*

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Proteolytic enzymes produced by *Porphyromonas gingivalis* are thought to play critical roles in the pathogenesis of periodontitis. The aim of this study was to investigate the effect of gingipain cysteine proteinase gene inactivation on selected pathological and physiological functions of *P. gingivalis*. Our results showed that Arg- and Lys-gingipain activities are critical components for the efficient growth of *P. gingivalis* in human serum. However, when the serum was supplemented with peptides provided as pancreatic casein hydrolysate, the gingipains did not appear to be essential for growth. The effect of gingipain gene inactivation on the susceptibility of *P. gingivalis* to serum bactericidal activity was investigated using standardized human serum. The wild-type strain, *P. gingivalis* ATCC 33277, was largely unaffected by the bactericidal activity of human serum complement. On the other hand, mutants lacking Arg-gingipain A, Arg-gingipain B, or Lys-gingipain activity were susceptible to complement. Since gingipains are mostly located on the outer membrane of *P. gingivalis*, inactivation of the genes for these enzymes may modify cell surface properties. We showed that gingipain-deficient mutants differed in their capacities to assimilate radiolabeled amino acids, cause hemolysis, express adhesins, hemagglutinate, and form biofilms. Lastly, the gingipains, more specifically Arg-gingipains, were responsible for causing major cell damage to human gingival fibroblasts. In conclusion, our study indicated that, in addition to being critical in the pathogenic process, gingipains may play a variety of physiological roles in *P. gingivalis*, including controlling the expression and/or processing of virulence factors. Mutations in gingipain genes thus give rise to pleiotropic effects.

Periodontitis is a group of inflammatory conditions with an infective etiology that leads to loss of tooth support. There is now a consensus that chronic periodontitis is initiated by several bacterial species that behave in a cooperative or synergistic fashion to produce the infection. A group of ~10 bacterial species, more particularly the highly proteolytic organisms *Porphyromonas gingivalis*, *Treponema denticola*, and *Bacteroides forsythus*, have been associated with chronic periodontitis (10, 25, 39, 40). Much evidence points to *P. gingivalis* as the key pathogen in chronic periodontitis. For example, studies have shown that *P. gingivalis* is detected with greater frequency and at higher levels at periodontal sites that appear to be disease active (33, 41) and that certain periodontal health indicators in individuals are inversely correlated with the presence or level of *P. gingivalis* (15, 16).

Virulence factors produced by *P. gingivalis* include outer membrane vesicles, adhesins, lipopolysaccharides, hemolysins, and proteinases (9, 13, 17, 18). Three different genes code for arginine-X (Arg-gingipain A and B [*rgpA* and *rgpB*])- and lysine-X (Lys-gingipain [*kgp*])-specific cysteine proteinases (31, 32), which occur in multiple molecular forms due to proteolytic processing of the initially translated polypeptides (7, 8, 23, 31). In addition, the genes *rgpA* and *kgp* contain a sequence encoding adhesion domains (8, 20, 34). Because of their broad activity spectrums, the RgpA, RgpB, and Kgp cysteine proteinases of *P. gingivalis* are thought to play critical roles in the pathogenesis of periodontitis, more particularly in host colonization, inactivation of host defenses, tissue destruction, and modulation of the host immune system (9, 18).

In addition to being critical in the pathogenic process, gingipains may play a variety of physiological roles in *P. gingivalis*, including control of the expression of virulence factors and of the stability and/or processing of extracellular or cell surface proteins. For example, Arg-gingipains (i) contribute to the maturation of the hemoglobin-binding receptor protein domain and the hemagglutinating activity of the *hagA* gene product; (ii) process an immunogenic 75-kDa cell surface protein, profimbrillin, and pro-Kgp; and (iii) participate in their own processing (20, 21, 30). In recent years, a number of research groups have constructed specific gingipain-deficient isogenic *P. gingivalis* mutants (1, 27, 37, 42, 45) which are of particular interest for investigating the biological and physiological functions of gingipains. In this study, we used a set of *P. gingivalis* mutants constructed by allelic replacement mutagenesis or integration of a suicide plasmid to investigate the effect of the inactivation of the Arg- and/or Lys-gingipain gene on selected virulence and physiological properties.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. We used *P. gingivalis* ATCC 33277 and five gingipain-deficient mutants constructed using allelic replacement mutagenesis or integration of a suicide plasmid (27, 37). KDP129 is a *kgp* (Lys-gingipain) mutant, KDP131 is an *rgpA* (Arg-gingipain A) mutant, KDP132 is an *rgpB* (Arg-gingipain B) mutant, KDP112 is an *rgpA rgpB* (Arg-gingipains A and B) double mutant, and KDP128 is a *kgp rgpA rgpB* (Lys-gingipain; Arg-gingipains A and B) triple mutant. The bacteria were grown in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) supplemented with 0.001% hemin and 0.0001% vitamin K (THBHK). To maintain selective pressure and prevent the appearance of revertants, antibiotics were added when the mutants KDP112 (tetracycline at 0.7 μg/ml and erythromycin at 10 μg/ml), KDP131 (erythromycin at 10 μg/ml), KDP132 (tetracycline at 0.7 μg/ml), and KDP128 (tetracycline at 0.7 μg/ml and erythromycin at 10 μg/ml) were grown on agar plates. To avoid possible side effects on the properties being studied, antibiotics were not added to broth media for preparing the cells to be used in the experiments described below. All cultures were incubated at 37°C under anaerobic conditions (N_2-H_2-CO_2 [80:10:10]). Prior to using mutants in the experiments, their phenotypes were confirmed by testing their abilities to cleave the chromogenic synthetic substrates for Arg-gingipain (benzoyl-Arg-*p*-nitroanilide) and Lys-gingipain (*N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide) as described previously (14).

Culture plate assay for the determination of general proteinase activity. Gelatin agar plates were prepared by adding 1% (wt/vol) gelatin (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and 3% (wt/vol) pancreatic hydrolysate of casein (tryptone; BBL Microbiology Systems, Cockeysville, Md.) to the chemically defined medium previously described by Milner et al. (24). This medium contains NaH_2PO_4 (10 mM), KCl (10 mM), $MgCl_2$ (1.2 mM), $ZnCl_2$ (25 mM), $CaCl_2$ (20 mM), $CoCl_2$ (10 mM), $CuCl_2$ (5 mM), $NaMoO_4$ (0.1 mM), boric acid (5 mM), citric acid (2 mM), α-ketoglutarate (50 mM), hemin (7.5 μM), and vitamin K (3 μM) and is adjusted to pH 7. Five microliters of 24-h bacterial cultures in THBHK was spotted on the surface of agar plates and allowed to dry for 10 min. The plates were inverted and incubated in the anaerobic chamber at 37°C for 7 days. They were then stained with 0.6% amido black in methanol-acetic acid-distilled water (45:10:45) for 30 min, destained with solvent (methanol-acetic acid-distilled water), and examined for clear zones, which indicated gelatin degradation, around the bacterial growth. The proteolysis zones were measured in millimeters from the edge of the growth to the outer margin of the clear zone. The mean and standard deviation of three independent experiments was calculated.

Growth in human serum. Human serum was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, Calif.), diluted 1:10 in sterile distilled water, and supplemented with either 0.01% hemin or 0.01% hemin plus 1% pancreatic hydrolysate of casein (tryptone; BBL Microbiology Systems). The serum was inoculated with a 10% inoculum of a 24-h subculture of *P. gingivalis* in THBHK and incubated at 37°C under anaerobiosis. Growth was monitored by recording the optical density at 660 nm (OD_{660}) every 2 h. Doubling times were then calculated by regression analysis of OD_{660} values from the exponential growth phase. Protein degradation during the growth of the wild-type *P. gingivalis* strain ATCC 33277 was analyzed by sodium dodecyl sulfate–11% polyacrylamide gel electrophoresis (SDS–11% PAGE) and immunoblotting. Briefly, an equal volume of denaturing-reducing sample buffer was added to each culture sample (1/100 dilution in distilled water) collected at various incubation times, and the mixtures were immediately boiled for 10 min. After electrophoretic separation, the proteins were transferred onto a nitrocellulose membrane, which was incubated with either alkaline phosphatase (AP)-conjugated goat anti-human albumin antibody (1/8,000 dilution), AP-conjugated chicken anti-human transferrin antibody (1/3,000 dilution), or AP-conjugated chicken anti-human immunoglobulin G (IgG) antibody (1/3,000 dilution). The antibodies were obtained from Bethyl Laboratories Inc. (Montgomery, Tex.). Undegraded proteins and proteolytic fragments were visualized following development in carbonate buffer (100 mM $NaHCO_3$–10 mM $MgCl_2$ [pH 9.8]) containing 0.3 mg of nitroblue tetrazolium chloride/ml and 0.15 mg of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt/ml.

Cellular uptake of radiolabeled amino acids. Twenty-four-hour *P. gingivalis* cultures (1 ml) were incubated with a mixture of ^{14}C-labeled amino acids (50 μCi/ml; Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) at a final concentration of 10 μCi/ml. After an incubation period of 4 h under anaerobic conditions at 37°C, the cells were harvested by centrifugation (10,000 × *g* for 10 min) and washed twice in cold 50 mM phosphate-buffered saline (PBS; pH 7.2). The cells were resuspended in cold PBS, and the radioactivity incorporated by bacterial cells was measured in a scintillation counter. The means and standard deviations of three independent experiments were calculated.

Resistance to serum bactericidal activity. Standardized human serum complement (Sigma Chemical Co., St. Louis, Mo.) was used to evaluate the susceptibilities of *P. gingivalis* ATCC 33277 and the gingipain-deficient mutants to the bactericidal activity of serum complement. Approximately 10^8 cells (determined using a Petroff-Hausser counting chamber) from a 24-h culture in THBHK were suspended in 200 μl of human serum complement (diluted 1/5 in sterile PBS). Rabbit anti-*P. gingivalis* serum produced in our laboratory (10 μl) was then added. Assays were also performed using heat-inactivated (56°C for 30 min) human serum complement. The mixtures were incubated at 37°C under anaerobiosis, and samples were taken at 0 and 2 h. Viable bacteria were determined by spreading serial dilutions (1:10) on THBHK plates supplemented with 2.5% (vol/vol) sheep blood. The percent viability after a 2-h incubation with the serum complement was established based on the viable counts of the time zero samples. The means and standard deviations of three independent experiments were calculated.

Hemagglutinating activity and expression of HAs. The hemagglutinating activity of *P. gingivalis* cells was measured as follows. Briefly, 100 μl of PBS was added to each well of a round-bottom microtiter plate. Then, 100 μl of the bacterial cells (OD_{660} = 1.0 in PBS) was added to the first well and serially diluted (1:2 to 1:128). Finally, 100 μl of 2.5% (vol/vol) washed sheep erythrocytes was added to each well, and the plate was incubated at room temperature for 3 h. The hemagglutinating activity was assessed visually, and the reciprocal of the highest dilution displaying a positive agglutination of erythrocytes was recorded. The expression of components (43- and 49-kDa polypeptides) of the hemagglutinating adhesin hemagglutinin (HA)-Ag2 was evaluated by SDS-PAGE and immunoblotting as previously described (2, 3, 4). Cells were grown in THBHK for 24 h, harvested by centrifugation, treated with 10% trichloroacetic acid (1 h at 25°C), and suspended in PBS at an OD_{660} of 1.0. The cell suspensions were mixed with equal volumes of denaturing-reducing sample buffer. The samples were boiled for 10 min, and the proteins were separated on an 11% polyacrylamide gel and transferred onto a nitrocellulose membrane. The 43- and 49-kDa polypeptides were detected using HA-Ag2-specific rabbit antiserum (1/5,000 dilution; primary antibody) and AP-conjugated IgG goat anti-rabbit (1/30,000 dilution; secondary antibody) as described previously (2, 3, 4).

Hemolytic activity. Fresh sheep erythrocytes were harvested from whole blood by centrifugation (600 × *g* for 5 min). They were washed three times in PBS and suspended in PBS to a concentration of 2% (vol/vol). Equal volumes (1 ml) of erythrocytes and bacterial cells (OD_{660} = 1.0 in PBS) were mixed together and incubated at 37°C for 4 h. PBS replaced the bacteria in the negative control. After the incubation, the mixtures were centrifuged (10,000 × *g* for 5 min) and the absorbance of the supernatants was measured at 540 nm. The means and standard deviations of four independent experiments were calculated.

Biofilm formation. A 24-h *P. gingivalis* THBHK culture was diluted 1/20 in reduced THB medium to obtain a final OD_{660} of 0.075. Samples (200 μl) were added to the wells of a 96-well flat-bottom polystyrene microtiter plate (Nalge Nunc, Naperville, Ill.). After cultivation for 48 h at 37°C under anaerobic conditions, free-floating bacteria were removed by aspiration using a 26-gauge needle. The wells were washed twice with 10 mM PBS, and the plate was inverted and dried. The bacterial biofilms were stained with 0.1% safranine for 15 min (100 μl per well). The plate was washed three times with distilled water and dried for 3 h at 37°C. After the addition of 100 μl of 95% (vol/vol) ethanol to each well, the plate was shaken for 15 min to release the stain from the biofilms, and the absorbance at 492 nm was recorded using an enzyme-linked immunosorbent assay reader. Assays were run in triplicate, and the means and standard deviations of four independent experiments were calculated.

Cytotoxic activity. The effect of gingipain gene inactivation on the cytotoxicity of *P. gingivalis* was determined by measuring the abilities of bacterial cell suspensions or bacterial culture supernatants to cause morphological changes and cell death of human gingival fibroblasts (HGFs) as previously described (26). HGFs were obtained by emigration on plastic dishes from a gingival biopsy specimen from a 55-year-old patient with a clinically healthy periodontium. The HGFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum at 37°C in a 5% CO_2 atmosphere. Cytotoxicity assays were performed with cells (passages 4 and 5) that were cultivated in a 24-well plate until a confluent monolayer was obtained. Bacteria from stationary-phase cultures were collected by centrifugation (10,000 × *g* for 10 min), washed twice in PBS, and adjusted to an OD_{660} of 0.25 in DMEM. An aliquot of each bacterial sample was heated at 100°C for 15 min to inactivate enzymes. Samples (250 μl) of bacterial cell suspensions or culture supernatants (diluted 20-fold in DMEM) mixed with an equal volume of DMEM were added to HGF monolayer cultures that had been washed three times with DMEM. Infected HGF cultures were incubated for 24 h in a CO_2 incubator, and morphological changes (cell rounding) were observed using an inverted microscope.

TABLE 1. Production of proteolysis zones on a gelatin plate and uptake of radiolabeled amino acids by *P. gingivalis* ATCC 33277 and gingipain-deficient mutants

Strain	Proteolysis zone (mm)[a]	Radioactivity incorporated (DPM)[b]
ATCC 33277	9 ± 0.5	26,522 ± 1,217
KDP131 (*rgpA*)	9 ± 0.5	48,696 ± 1,522*
KDP132 (*rgpB*)	12 ± 1	45,652 ± 739*
KDP112 (*rgpA rgpB*)	3 ± 1*	54,348 ± 3,043*
KDP129 (*kgp*)	11 ± 0.5	52,391 ± 1,323*
KDP128 (*rgpA rgpB kgp*)	0*	66,087 ± 870*

[a] Zones were measured from the edge of the bacterial growth to the outer margin of the clear zone. Shown are means ± standard deviations of three independent experiments. The asterisks indicate a significant difference with respect to the wild-type strain ($P < 0.005$).

[b] Means ± standard deviations of three independent experiments. The asterisks indicate a significant difference with respect to the wild-type strain ($P < 0.005$).

The ratio of cell rounding was calculated from a photograph of the culture. After the supernatant was removed, the HGFs were collected by treating the monolayers with PBS containing 0.01% trypsin and 0.02% EDTA (37°C for 5 min) and were stained with 0.4% trypan blue. Viable adherent and nonadherent cells were counted using a hemocytometer, and the percent viability was calculated. Analyses were performed twice.

Statistical analysis. Significant differences between the means (± standard deviations) were determined using a two-tailed Student's *t* test. Differences in *P* values of <0.005 were considered significant.

RESULTS

Gingipain-deficient mutants derived from *P. gingivalis* ATCC 33277 were first tested in a culture plate assay for the capacity to degrade gelatin, a general substrate susceptible to most proteolytic enzymes (Table 1). Mutant KDP132 (deficient in *rgpB*) and mutant KDP129 (deficient in *kgp*) produced larger proteolysis zones than the wild-type strain. When these two mutants were tested using specific chromogenic substrates for gingipains, it was found that mutant KDP132 overexpresses extracellular Lys-gingipain activity (3.6 times that of the parent strain) and mutant KDP129 overexpresses extracellular Arg-gingipain activity (1.7 times that of the parent strain) (data not shown). Inactivation of *rgpA* (mutant KDP131) did not affect the size of the proteolysis zone. On the other hand, mutant KDP112 (deficient in both *rgpA* and *rgpB*) produced a smaller zone than the parent strain, whereas the triple mutant KDP128 (deficient in *kgp* and in *rgpA* and *rgpB*) did not produce a clear zone. The differences in the sizes of the proteolysis zones were highly reproducible and could not be attributed to differences in growth, since all of the mutants grew to the same extent as the wild-type strain. This quantification was done by measuring the OD_{660} of the suspensions of cells harvested from the surfaces of the plates.

The effect of gingipain gene inactivation on the capacity of *P. gingivalis* to multiply in human serum, whether supplemented or not with a pancreatic hydrolysate of casein as a source of peptides, was determined (Table 2). When no casein hydrolysate was added to human serum, only the wild-type strain (ATCC 33277) and the mutant deficient in *rgpB* (KDP132) grew. However, the wild-type ATCC 33277 strain had a much shorter doubling time and reached a higher OD_{660} after 36 h than mutant KDP132. The doubling time of the wild-type strain in the absence of casein hydrolysate was comparable to that obtained in human serum supplemented with peptides (casein hydrolysate). All the strains grew well in the presence of casein hydrolysate and reached OD_{660}s ranging from 0.45 to 0.57 after 36 h of incubation. The triple mutant KDP128 (*rgpA rgpB kgp*) had the longest doubling time (6.7 h versus 3.8 h for the wild-type strain). SDS-PAGE–Western immunoblotting analysis of culture samples of *P. gingivalis* ATCC 33277 grown in human serum was performed to evaluate the preference of the bacteria for specific serum proteins (data not shown). *P. gingivalis* efficiently degraded, with no particular preference, albumin, transferrin, and IgG during growth in human serum without the peptide supplement. After 36 h of incubation, most proteins were degraded into fragments not detectable by SDS-PAGE–Western immunoblotting. When *P. gingivalis* was grown in human serum supplemented with casein hydrolysate, the degradation of serum proteins was much less pronounced, particularly at 12 h (data not shown).

TABLE 2. Growth in human serum and complement susceptibility of *P. gingivalis* ATCC 33277 and gingipain-deficient mutants

Strain	Growth (OD_{660}[a]/doubling time [h])		% Viability in[b]:	
	Serum + hemin	Serum + hemin + casein hydrolysate	Serum	Inactivated serum
ATCC 33277	0.33/4	0.57/3.8	87 ± 11	92 ± 7
KDP131 (*rgpA*)	NSG[c]	0.56/5.4	ND[d]	ND
KDP132 (*rgpB*)	0.16/9.3	0.48/4.2	ND	ND
KDP129 (*kgp*)	NSG	0.45/5.1	22 ± 9*	72 ± 12
KDP112 (*rgpA rgpB*)	NSG	0.56/4.8	28 ± 14*	82 ± 10
KDP128 (*rgpA rgpB kgp*)	NSG	0.48/6.7	0*	0*

[a] OD obtained after 36 h of growth.

[b] Percent viability after the 2-h treatment was calculated as a function of the viable counts obtained with the time zero samples. Shown are means ± standard deviations of three independent experiments. The asterisks indicate a significant difference with respect to the wild-type strain ($P < 0.005$).

[c] NSG, no significant growth.

[d] ND, not determined.

The effect of gingipain gene inactivation on the susceptibility of *P. gingivalis* to serum bactericidal activity was investigated using a standardized human serum containing all active components of the complement system. Prior to performance of the assays, antibodies to *P. gingivalis* were added to activate the complement by the classic pathway. The wild-type strain (*P. gingivalis* ATCC 33277) was relatively unaffected by the bactericidal activity of human serum complement (Table 2). On the other hand, mutants lacking either Arg-gingipain activities A and B or Lys-gingipain had similar, higher levels of susceptibility ($P < 0.005$). The decrease in cell viability was complement dependent, since no such loss of viability was observed with heat-inactivated serum. The mutant KDP128 (*rgpA rgpB kgp*) was killed by both active and heat-inactivated serum.

Since gingipains are mostly located on the outer membrane of *P. gingivalis*, inactivation of the genes coding for these enzymes may modify cell surface properties. To investigate this possibility, the rates of uptake of radiolabeled amino acids by the wild-type strain and the mutants were determined. Table 1

TABLE 3. Activities of hemagglutination, biofilm formation, and hemolysis for *P. gingivalis* ATCC 33277 and gingipain-deficient mutants

Strain	Hemagglutination[a] (assay 1/assay 2)	Biofilm formation[b] (A_{592})	Hemolysis[c] (A_{540})
ATCC 33277	8/16	0.35 ± 0.01	1.1 ± 0.15
KDP131 (*rgpA*)	4/8	0.56 ± 0.03*	ND[d]
KDP132 (*rgpB*)	8/16	0.32 ± 0.01	ND
KDP129 (*kgp*)	2/4	0.36 ± 0.01	1.76 ± 0.05*
KDP112 (*rgpA rgpB*)	−	0.28 ± 0.02	0.73 ± 0.12
KDP128 (*rgpA rgpB kgp*)	−	0.21 ± 0.01	0.83 ± 0.09

[a] Reciprocal of the highest dilution producing hemagglutination. −, negative.
[b] Means ± standard deviations of four assays. The asterisk indicates a significant difference with respect to the wild-type strain ($P < 0.005$).
[c] Complete lysis of erythrocytes caused by adding 1% SDS gave an A_{540} of 1.8. The mean baseline value of the assay was 0.048. The asterisk indicates a significant difference with respect to the wild-type strain ($P < 0.005$).
[d] ND, not determined.

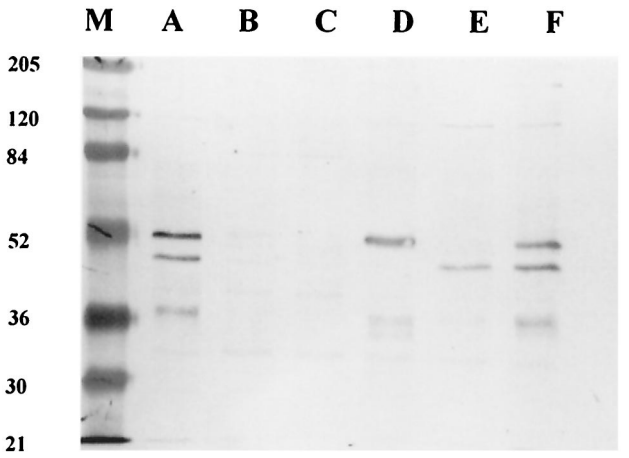

FIG. 1. SDS-PAGE and Western immunoblotting of cell lysates of *P. gingivalis* ATCC 33277 and the gingipain-deficient mutants using an antiserum directed against the HA-Ag2 antigen of *P. gingivalis*. Lane M, molecular weight markers; lane A, ATCC 33277; lane B, KDP112; lane C, KDP128; lane D, KDP129; lane E, KDP131; lane F, KDP132.

shows that all mutants incorporated more radioactivity than the wild-type strain. The largest difference was observed with the triple mutant, KDP128 (*rgpA rgpB kgp*), which incorporated ~2.5-fold more radiolabeled amino acids than the wild-type strain.

The hemagglutinating activities of *P. gingivalis* ATCC 33277 and the mutants were also determined (Table 3). Mutants KDP112 (*rgpA rgpB*) and KDP128 (*rgpA rgpB kgp*) were unable to agglutinate erythrocytes, while mutant KDP132 (*rgpB*) displayed normal activity and mutants KDP131 (*rgpA*) and KDP129 (*kgp*) had decreased capacities to agglutinate sheep erythrocytes. Different hemaggulutinins of *P. gingivalis* with different molecular weights and properties have been reported by different research groups. HA-Ag2 that we previously reported is characterized by the presence of two bands at 43 and 49 kDa when resolved on SDS–12% PAGE (3, 4). In order to elucidate the relationship of HA-Ag2 with the genetically well-characterized gingipains, and to analyze the expression of the two HA-Ag2 bands in *P. gingivalis* mutants, cell lysates from wild-type and mutant strains of *P. gingivalis* were separated on SDS-PAGE, electrotransferred to nitrocellulose membranes, and revealed by a monospecific antiserum against HA-Ag2. Cell lysates of strains ATCC 33277 and mutant KDP132 contained both the 43- and 49-kDa polypeptides, which are components of the hemagglutinating adhesin HA-Ag2 (Fig. 1). On the other hand, neither polypeptide was detected in KDP112 and KDP128 lysates, while only the 43-kDa polypeptide was present in the lysate of mutant KDP131 and only the 49-kDa polypeptide was present in the lysate of mutant KDP129. The effect of an Arg- and/or Lys-gingipain deficiency on the hemolysis of sheep erythrocytes by *P. gingivalis* was determined. As shown in Table 3, mutant KDP129, which lacks Lys-gingipain activity, displayed more hemolytic activity than the wild-type strain. Mutants (KDP112 and KDP128), with no Arg-gingipain activity, had reduced capacities to lyse erythrocytes.

A microtiter plate assay was used to investigate biofilm formation by *P. gingivalis* and the gingipain-deficient mutants. Table 3 shows that mutant KDP131 (*rgpA*) produced more biofilm while mutants KDP112 (*rgpA rgpB*) and KDP128 (*rgpA rgpB kgp*) produced less biofilm than the wild-type strain.

Figure 2 shows the morphological changes in HGFs infected with *P. gingivalis*. Almost all the HGFs incubated with *P. gingivalis* ATCC 33277 and KDP129 (deficient in Lys-gingipain activity) were rounded (97.8 and 89.5%, respectively). Although some cells lacked adherence capacity and remained free floating, most (76% for ATCC 33277 and 82% for KDP129) remained attached to the plastic surface. The results of the trypan blue exclusion test showed that ~95% of the floating cells were dead, whereas >99% of the attached cells were viable. Mutant KDP112 caused only slight morphological changes (narrow shape) but did not lead to cell rounding or cell death. Inactivation of only one *rgp* gene (A or B) showed results comparable to those obtained with the wild-type strain (data not shown). Mutant KDP128, as well as boiled cells of the other strains tested, had no effect on the morphology and viability of HGFs. ATCC 33277 and KDP129 culture supernatants caused HGFs to detach from the plastic. The KDP129 supernatant produced a stronger effect than the ATCC 33277 supernatant (75% detachment for KDP129 and 25% for ATCC 33277). These effects were consistent with the difference in benzoyl-Arg-*p*-nitroanilide-hydrolyzing activity of the supernatants (KDP129 versus ATCC 33277, 2.1:1). As with the bacterial cells, the culture supernatants of strains KDP112 and KDP128 did not produce any significant changes in the morphology of HGFs.

DISCUSSION

Bacterial proteinases have a variety of pathological and physiological functions. Thus, inactivation of proteinase genes may result in a variety of effects. Inactivation of the *Staphylococcus aureus* SspA serine protease (also called V8 protease) causes a pleiotropic effect on the profile of secreted proteins, including autolysin activity, and on the proteolytic maturation of the SspB cysteine protease (35). Recently, Woischnik et al. (43) reported that inactivation of the *Streptococcus pyogenes* SpeB cysteine protease decreases hyaluronic acid capsule expression and increases the production of superoxide dismutase. In *P. gingivalis*, studies of the pleiotropic effect of proteinase gene inactivation have been limited to HAs and

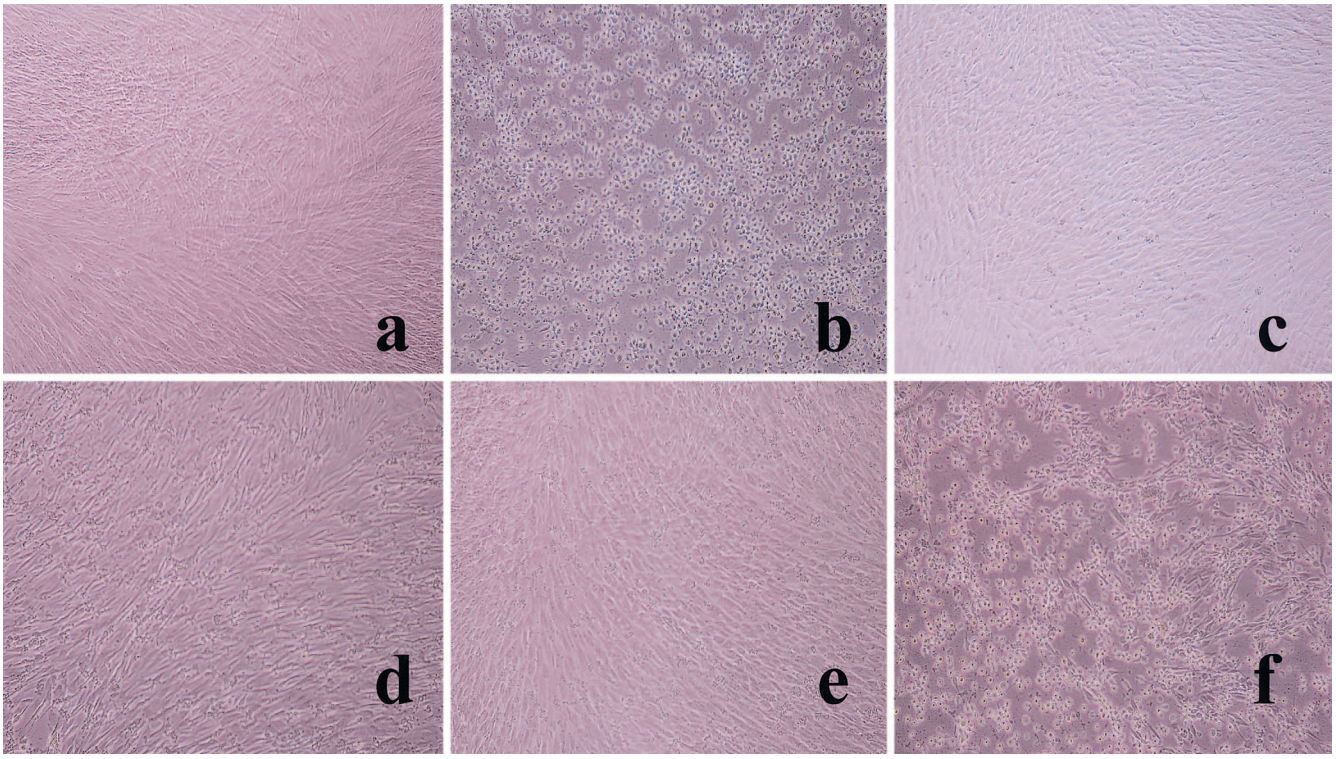

FIG. 2. Morphological changes observed in human gingival fibroblasts infected with *P. gingivalis* ATCC 33277 and gingipain-deficient mutants. (a) Control without bacteria; (b) ATCC 33277; (c) heat-treated ATCC 33277; (d) KDP112; (e) KDP128; (f) KDP129.

fimbriae (28, 42, 44). The aim of our study was to investigate the effects of gingipain gene inactivation on selected pathological and physiological properties of *P. gingivalis*.

The effect of gingipain gene inactivation on the general proteolytic activity of *P. gingivalis* was investigated using a gelatin plate assay. Mutations in either *rgpB* or *kgp* resulted in increased proteolytic activity, as shown by the larger zones obtained for mutants KDP132 and KDP129 compared to the wild-type strain. Further analysis using specific chromogenic substrates for gingipains revealed that these mutants overexpress extracellular Lys-gingipain activity and extracellular Arg-gingipain activity, respectively. The fact that no proteolysis zone was produced by mutant KDP128 indicated that RgpA, RgpB, and Kgp cysteine proteinases are responsible for most *P. gingivalis* endoproteinase activity. The absence of proteolysis may also be explained by the fact that RgpA, RgpB, and/or Kgp are essential for processing other nongingipain proteinases.

Human serum supported the growth of *P. gingivalis*. It mimics in vivo conditions, since gingival crevicular fluid, a serum derivative, bathes the periodontal pocket. Our results indicated that Arg- and Lys-gingipain activities were critical for efficient growth of *P. gingivalis* in human serum. On the other hand, when the serum was supplemented with peptides (pancreatic hydrolysate of casein), the gingipains were not essential for growth. However, the doubling time for mutant KDP128 (deficient in both Arg- and Lys-gingipains) was longer than that of the wild-type strain (6.7 versus 3.8 h), suggesting that gingipains, although not essential, may promote growth by producing assimilable peptides. This supports a recent study reporting that the growth rates of gingipain-deficient mutants and the parent *P. gingivalis* ATCC 33277 strain were similar when a chemically defined medium was supplemented with a protein hydrolysate instead of human serum albumin (14). SDS-PAGE and Western immunoblotting showed that supplementing the human serum with peptides delayed the degradation of serum proteins (albumin, transferrin, and IgG) during the growth of the wild-type *P. gingivalis*. This suggests that the added peptides might be preferentially used for growth and that serum proteins are not necessarily used as nutrients. Our study revealed that the inactivation of gingipain genes resulted in an increased capacity to assimilate radiolabeled amino acids, suggesting that mutations might increase either the permeability of the bacterial cell envelope or the number of specific amino acid transporters. It is also possible that, as with *S. pyogenes* (43), gene inactivation results in a loss of capsular material, thus increasing the nutrient transport capacity of the bacterium.

Previous studies (12, 36) have shown that several components of the complement system are susceptible to degradation by *P. gingivalis*. In this study, we clearly showed that both Lys- and Arg-gingipain activities were required for optimal bacterial resistance to the bactericidal activity of human serum. The mutant KDP128 (*rgpA rgpB kgp*) was highly sensitive to even heat-inactivated serum complement. This may be related to the fact that, in the absence of gingipain activity, the iron-binding proteins in serum remain undegraded and may damage the outer bacterial membrane and alter its permeability (11).

Our data confirmed previous studies indicating that Arg-gingipain gene inactivation results in decreased hemagglutinat-

ing activity by *P. gingivalis* (22, 37). It has been reported that the hemagglutinating adhesin HA-Ag2 possesses antigenic, structural, and functional relationships with the fimbriae of *P. gingivalis* (3, 4). However, until now there were no data on the nature of the relationship between HA-Ag2 and the gingipains. In this study, using a specific antibody, we produced evidence for the first time that the 43- and 49-kDa polypeptides may be related to the HA domains of the genes *kgp* and *rgpA*, respectively. Shi et al. (37) proposed two hypotheses regarding the involvement of Arg-gingipains in hemagglutination. First, the maturation of adhesins may require the participation of these enzymes, and second, Arg-gingipains may modify erythrocyte surface molecules, thus increasing the binding activity of *P. gingivalis* cells. Chen et al. (5) constructed Lys-gingipain-deficient mutants of *P. gingivalis* by transposon insertion. The mutants produced white pigmentation on blood agar plates, displayed more Arg-gingipain activity, and produced more fimbrillin and fimbriae, resulting in a greater capacity to adhere to epithelial cells. On the other hand, the mutants had reduced capacity to agglutinate, to lyse red blood cells, and to produce outer membrane vesicles. Lewis et al. (22) also reported that mutations abolishing the activity of Lys-gingipain resulted in a loss of black pigmentation, as well as decreased hemagglutinating and hemolytic activities compared to the wild-type strain. Surprisingly, the Lys-gingipain mutant KDP129 used in our study had higher hemolytic activity. These contradictory results may be due to the methodology (hemolytic assay and origin of erythrocytes) or the fact that the mutants were not constructed from the same wild-type strain. The apparently higher hemolytic activity of mutant KDP129, with respect to the parent strain, may be explained by the fact that expression of the *kgp* gene results in a cell surface hemoglobin-binding activity (29), which could reduce the amount of free hemoglobin in the supernatant of the hemolytic assay. The fact that mutant KDP128, which produces no gingipains, displayed hemolytic activity suggests that gingipains may not be solely responsible for the lysis of erythrocytes.

Chen et al. (6) investigated the adherence of Arg-gingipain-deficient mutants of *P. gingivalis* ATCC 33277 to epithelial cells. They found that greater numbers of the KDP112 *rgpA rgpB* mutant than of the wild-type strain attached to KB oral epithelial cells. They suggested that when the catalytic activity of Arg-gingipain is reduced by mutation, more bacteria attach (via Kgp or HagA adhesin peptides) and accumulate on the monolayers but do not detach when the binding substrate is digested. A mutant of strain 381 defective in the *rgpA* gene was also reported to attach more efficiently to cultured epithelial cells than the parent strain (42). Mutant KDP112 (*rgpA rgpB*) possesses very few fimbriae, suggesting that a protein other than fimbrillin may function as an adhesin for attachment to epithelial cells (28). Contradictory results have been reported concerning the effect of a single mutation in *rgpA* on fimbriation. Nakayama et al. (28) reported that fimbriation is normal in an *rgpA* mutant, whereas Tokuda et al. (42) and Xie et al. (44) reported that inactivation of the RgpA proteinase gene results in a significantly increased expression of fimbriae. The ability of bacteria to grow in a biofilm is likely related to the composition and charge of the bacterial cell surface. We showed that the lack of certain gingipains can result in either an increased or a decreased capacity to form a biofilm. Some gingipains may uncover bacterial coaggregation factors, thus decreasing the capacity of bacteria to aggregate, while others may have a negative effect by degrading these coaggregation factors.

The toxicity of *P. gingivalis* for HGFs has been reported (19, 26). Based on the results obtained with cysteine proteinase inhibitors, the cytotoxic effect may be caused by this class of proteases. In the present study, we clearly showed that gingipains, more specifically Arg-gingipains, are responsible for the major cell-damaging effect on HGFs. The contribution of *P. gingivalis* lipopolysaccharides and metabolic end products may thus be minor compared to gingipains.

Shoji et al. (38) recently described the pleiotropic phenotype of a *porR* mutant of *P. gingivalis*, including delayed maturation of fimbrillin, preferential presence of Rgp and Kgp proteinases in the culture supernatant, and no hemagglutinating activity. Shoji et al. (38) suggested that the *porR* gene may be involved in the biosynthesis of cell surface polysaccharides that may function as anchors for several cell surface molecules. We report here several pleiotropic effects associated with gingipain gene inactivation in *P. gingivalis*. We intend to conduct a complete analysis of these pleiotropic effects at the molecular level using proteomic technology. The mutants and parent strains will be compared by two-dimensional polyacrylamide gel electrophoresis, proteins whose expression is significantly modified (positively or negatively) will be analyzed by mass spectrometric peptide analysis, and protein database searches will be performed to identify them. This will enable us to identify and characterize the structures and functions of *P. gingivalis* proteins that are affected by the inactivation of a specific gingipain.

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REFERENCES

1. **Aduse-Opoku, J., M. Rangarajan, K. A. Young, and M. A. Curtis.** 1998. Maturation of the arginine-specific proteases of *Porphyromonas gingivalis* W50 is dependent on a functional *prR2* protease gene. Infect. Immun. **66:** 1594–1600.
2. **Chandad, F., and C. Mouton.** 1990. Molecular size variation of the hemagglutinating adhesin HA-Ag2, a common antigen of *Bacteroides gingivalis*. Can. J. Microbiol. **36:**690–696.
3. **Chandad, F., and C. Mouton.** 1995. Antigenic, structural, and functional relationship between fimbriae and the hemagglutinating adhesin HA-Ag2 of *Porphyromonas gingivalis*. Infect. Immun. **63:**4755–4763.
4. **Chandad, F., D. Mayrand, D. Grenier, D. Hinode, and C. Mouton.** 1996. Selection and phenotypic characterization of nonhemagglutinating mutants of *Porphyromonas gingivalis*. Infect. Immun. **64:**952–958.
5. **Chen, T., H. Dong, R. Yong, and M. J. Duncan.** 2000. Pleiotropic pigmentation mutants of *Porphyromonas gingivalis*. Microb. Pathog. **28:**235–247.
6. **Chen, T., K. Nakayama, L. Belliveau, and M. J. Duncan.** 2001. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. Infect. Immun. **69:**3048–3056.
7. **Curtis, M. A., H. K. Kuramitsu, M. Lantz, F. L. Macrina, K. Nakayama, J. Potempa, E. C. Reynolds, and J. Aduse-Opoku.** 1999. Molecular genetics and nomenclature of proteases of *Porphyromonas gingivalis.* J. Periodont. Res. **34:**464–472.
8. **Curtis, M. A., A. Thickett, J. M. Slaney, M. Rangarajan, J. Aduse-Opoku, P. Shepherd, N. Paramonov, and E. F. Hounsell.** 1999. Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. Infect. Immun. **67:**3816–3823.
9. **Cutler, C. W., J. R. Calmer, and C. A. Genco.** 1995. Pathogenic strategies of the oral anaerobe *Porphyromonas gingivalis*. Trends Microbiol. **3:**45–51.
10. **Dzink, J. L., S. S. Socransky, and A. D. Haffajee.** 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. **15:**316–323.
11. **Ellison, R. T., III, T. J. Giehl, and F. M. LaForce.** 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. **56:**2774–2781.

12. **Grenier, D.** 1992. Inactivation of human serum bactericidal activity by a trypsinlike protease isolated from *Porphyromonas gingivalis*. Infect. Immun. **60:**1854–1857.
13. **Grenier, D., and D. Mayrand.** 2000. Periodontitis as an ecological imbalance, p. 275–311. *In* H. K. Kuramitsu and R. Ellen (ed.), Oral bacterial ecology: the molecular basis. Horizon Scientific Press, Wymondham, Norfolk, United Kingdom.
14. **Grenier, D., S. Imbeault, P. Plamondon, G. Grenier, K. Nakayama, and D. Mayrand.** 2001. Role of gingipains in growth of *Porphyromonas gingivalis* in the presence of human serum albumin. Infect. Immun. **69:**5166–5172.
15. **Griffen, A. L., M. R. Becker, S. R. Lyons, M. L. Moeschberger, and E. J. Leys.** 1998. Prevalence of *Porphyromonas gingivalis* and periodontal health status. J. Clin. Microbiol. **36:**3239–3242.
16. **Grossi, S. G., J. J. Zambon, A. W. Ho, G. Koch, R. G. Dunford, E. E. Machtei, O. M. Norderyd, and R. J. Genco.** 1994. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J. Periodontol. **65:**260–267.
17. **Holt, S. C., and T. E. Bramanti.** 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit. Rev. Oral Biol. Med. **2:**177–281.
18. **Holt, S. C., L. Kesavalu, S. Walker, and C. A. Genco.** 1999. Virulence factors of *Porphyromonas gingivalis*. Periodontol. 2000 **20:**168–238.
19. **Johansson, A., and S. Kalfas.** 1998. Characterization of the proteinase-dependent cytotoxicity of *Porphyromonas gingivalis*. Eur. J. Oral Sci. **106:** 863–871.
20. **Kadowaki, T., K. Nakayama, K. Okamoto, N. Abe, A. Baba, Y. Shi, D. B. Ratnayake, and K. Yamamoto.** 2000. *Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. J. Biochem. **128:**153–159.
21. **Kadowaki, T., K. Nakayama, F. Yoshimura, K. Okamoto, N. Abe, and K. Yakamoto.** 1998. Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*. J. Biol. Chem. **273:**29072–29076.
22. **Lewis, J. P., J. A. Dawson, J. C. Hannis, D. Muddiman, and F. L. Macrina.** 1999. Hemoglobinase activity of the lysine gingipain protease (Kgp) of *Porphyromonas gingivalis* W83. J. Bacteriol. **181:**4905–4913.
23. **Mikolajczyk-Pawlinska, J., T. Kordula, N. Pavloff, P. A. Pemberton, W. C. Chen, J. Travis, and J. Potempa.** 1998. Genetic variation of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. Biol. Chem. **237:**205–211.
24. **Milner, P., J. Batten, and M. A. Curtis.** 1996. Development of a simple chemically defined medium for *Porphyromonas gingivalis*: requirement for α-ketoglutarate. FEMS Microbiol. Lett. **140:**125–130.
25. **Moore, W. E. C., L. V. Holdeman, R. M. Smibert, D. E. Hash, J. A. Burmeister, and R. R. Ranney.** 1982. Bacteriology of severe periodontitis in young adult humans. Infect. Immun. **38:**1137–1148.
26. **Morioka, M., D. Hinode, A. Nagata, H. Hayashi, S. Ichimiya, M. Ueda, R. Kido, and R. Nakamura.** 1993. Cytotoxicity of *Porphyromonas gingivalis* toward cultured human gingival fibroblasts. Oral Microbiol. Immunol. **8:**203–207.
27. **Nakayama, K., T. Kadowaki, K. Okamoto, and K. Yamamoto.** 1995. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis.* J. Biol. Chem. **270:** 23619–23626.
28. **Nakayama, K., F. Yoshimura, T. Kadowaki, and K. Yamamoto.** 1996. Involvement of arginine-specific cysteine proteinase (Arg-gingipain) in fimbriation of *Porphyromonas gingivalis*. J. Bacteriol. **178:**2818–2824.
29. **Okamoto, K., K. Nakayama, T. Kadowaki, N. Abe, D. B. Ratnayake, and K. Yamamoto.** 1998. Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. J. Biol. Chem. **273:**21225–21231.
30. **Potempa, J., A. Banbula, and J. Travis.** 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. Periodontol. 2000 **24:**153–192.
31. **Potempa, J., N. Pavloff, and J. Travis.** 1995. *Porphyromonas gingivalis*: a proteinase/gene accounting audit. Trends Microbiol. **3:**430–433.
32. **Potempa, J., R. Pike, and J. Travis.** 1995. Host and *Porphyromonas gingivalis* proteinases (gingipains) in periodontitis: a biochemical model of infection and tissue destruction. Perspect. Drug Discov. Des. **2:**445–458.
33. **Preus, H. R., A. Anerud, H. Boysen, R. G. Dunford, J. J. Zambon, and H. Loe.** 1995. The natural history of periodontal disease. The correlation of selected microbiological parameters with disease severity in Sri Lankan tea workers. J. Clin. Periodontol. **22:**674–678.
34. **Rangarajan, M., J. Aduse-Opoku, J. M. Slaney, K. A. Young, and M. A. Curtis.** 1997. The *prpR1* and *prR2* arginine-specific protease genes of *Porphyromonas gingivalis* W50 produce five biochemically distinct enzymes. Mol. Microbiol. **23:**955–965.
35. **Rice, K., R. Peralta, D. Bast, J. de Azavedo, and M. J. McGavin.** 2001. Description of staphylococcus serine protease (*ssp*) operon in *Staphylococcus aureus* and nonpolar inactivation of *sspA*-encoded serine protease. Infect. Immun. **69:**159–169.
36. **Schenkein, H. A.** 1988. The effect of periodontal proteolytic *Bacteroides* species on proteins of the human complement system. J. Periodont. Res. **23:**187–192.
37. **Shi, Y., D. B. Ratnayake, K. Okamoto, N. Abe, K. Yamamoto, and K. Nakayama.** 1999. Genetic analyses of proteolysis, hemoglobin-binding, and hemagglutination of *Porphyromonas gingivalis*: construction of mutants with a combination of *rgpA*, *rgpB*, *kgp* and *hagA.* J. Biol. Chem. **274:**17955–17960.
38. **Shoji, M., D. B. Ratnayake, Y. Shi, T. Kadowaki, K. Yamamoto, F. Yoshimura, A. Akamine, M. A. Curtis, and K. Nakayama.** 2002. Construction and characterization of a non-pigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. Microbiology **148:**1183–1191.
39. **Socransky, S. S., and A. D. Haffajee.** 1992. The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol. **63:**322–331.
40. **Socransky, S. S., A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent.** 1998. Microbial complexes in subgingival plaque. J. Periodontol. **25:**134–144.
41. **Socransky, S. S., A. D. Haffajee, C. Smith, and S. Dibart.** 1991. Relation of counts of microbial species to clinical status at the sampled sites. J. Clin. Periodontol. **18:**766–775.
42. **Tokuda, M., T. Karunakaran, M. Duncan, N. Hamada, and H. Kuramitsu.** 1998. Role of Arg-gingipain in virulence of *Porphyromonas gingivalis*. Infect. Immun. **66:**1159–1166.
43. **Woischnik, M., B. A. Buttaro, and A. Podbielski.** 2000. Inactivation of the cysteine protease SpeB affects hyaluronic acid capsule expression in group A streptococci. Microb. Pathog. **28:**221–226.
44. **Xie, H., W. O. Chung, Y. Park, and R. J. Lamont.** 2000. Regulation of the *Porphyromonas gingivalis fimA* (fimbrillin*)* gene. Infect. Immun. **68:**6574–6579.
45. **Yoneda, M., and H. K. Kuramitsu.** 1996. Genetic evidence for the relationship of *Porphyromonas gingivalis* cysteine protease and hemagglutinin activities. Oral Microbiol. Immunol. **11:**129–134.

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